

Heat Shock Induces Protein Tyrosine Phosphorylation in Cultured Cells

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Abstract. We examined the effect of heat shock on protein tyrosine phosphorylation in cultured animal cells using antiphosphotyrosine antibodies in immunoblotting and immunofluorescence microscopy experiments. Heat shock significantly elevated the level of phosphotyrosine in proteins in most of the cultured cells examined, including fibroblasts, epithelial cells,

nerve cells, and muscle cells, but not in Rous sarcoma virus-transformed fibroblasts. The increase in protein tyrosine phosphorylation induced by heat shock occurred in proteins with a wide range of molecular masses and was dependent on the temperature and duration of the heat shock.

THE exposure of cultured cells to elevated temperatures can result in a number of significant alterations in cellular metabolism. The most prominent change is a sharp reduction in normal protein synthesis, followed by induction of the synthesis of a small group of proteins known collectively as the heat-shock proteins (for reviews see Craig, 1985; Lindquist, 1986; Subjeck and Shyy, 1986). Heat shock can also stimulate phosphoinositide turnover (Calderwood et al., 1987) and the production of elevated levels of the *c-fos* protooncogene (Andrews et al., 1987). In addition, heat shock has previously been shown to alter the phosphorylation of some cellular proteins in several different systems, including soybean seedlings (Krishnan and Pueppke, 1987) and mammalian cell lines (Landry et al., 1988). However, the amino acids that are phosphorylated have not been determined.

We decided to examine the effect of heat shock on protein tyrosine phosphorylation in a variety of cultured cell lines using antibodies specific for phosphorylated tyrosine residues (Wang, 1985). We found that heat shock elevates the level of protein phosphorylation on tyrosine in many different types of cultured animal cells, including fibroblasts, epithelial-like cells, nerve cells, and muscle cells. The increase in protein tyrosine phosphorylation induced by heat shock is dependent on the temperature and duration of the heat shock. In all the cell types examined, the heat-shock-induced tyrosine phosphorylations occur in proteins with a wide range of molecular masses (30–200 kD).

Materials and Methods

Cell Culture

Swiss 3T3 fibroblasts and Rous sarcoma virus-transformed 3T3 fibroblasts

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were grown in DME with 10% FCS. The Swiss 3T3 fibroblasts were made quiescent as previously described (Pasquale et al., 1988). Chicken embryo fibroblasts were prepared by trypsinization of decapitated 8-d-old chick embryos and cultured in DME/Coon's F12 (1:1) plus 10% FCS. Madin-Darby canine kidney, PTK₂, L6, and neuroblastoma N2A cells were grown in MEM with 10% FCS. NRK cells and REF-52 cells were cultured in Coon's F12 with 10% FCS. PC12 cells were grown in DME plus 10% FCS and 5% horse serum. All cells were maintained at 37°C. To induce heat shock the cells were incubated in a water bath adjusted to 40–49°C. For the time course studies the culture medium was prewarmed to the desired temperature.

SDS-PAGE and Immunoblotting

Cells in 35-mm tissue culture dishes were solubilized in 100 μ l SDS sample buffer containing 1 mM PMSF (Sigma Chemical Co., St. Louis, MO), 0.2 TIU aprotinin/ml (Sigma Chemical Co.), and 1 mM sodium orthovanadate, sonicated, and boiled for 3 min. Equal amounts of protein (100 μ g/lane) were applied to a 7.5% SDS-polyacrylamide gel prepared as described (Laemmli, 1970). The proteins were transferred to nitrocellulose (Towbin et al., 1979), briefly stained with Amido black, and briefly destained with 10% methanol, 10% acetic acid to confirm the presence of equal amounts of cellular protein in all lanes. The transfers were incubated for at least 2 h in 3% BSA in TBS (BSA/TBS) and incubated for 4 h with 2 μ g/ml rabbit antiphosphotyrosine antibodies (Wang, 1985; Maher and Pasquale, 1988) in BSA/TBS. After washing with TBS, the immunoblots were incubated for 1 h with ¹²⁵I-protein A (0.25 μ Ci/ml; ICN Biomedicals Inc., Irvine, CA) in BSA/TBS, washed in TBS followed by 0.2% NP-40, 0.5% BSA in TBS, dried, and autoradiographed. The molecular mass standards used were filamin (250 kD, not shown), myosin (200 kD), vinculin (120 kD), α -actinin (100 kD), BSA (68 kD), and actin (43 kD).

Phosphoamino Acid Analysis

Cells were labeled for 3 h at 37°C with [³²P]orthophosphate (1 mCi/ml; Amersham Chemical Corp., Arlington Heights, IL) in phosphate-free DME containing 5% phosphate-free FCS and then left at 37°C for an additional hour or exposed at 46°C for 1 h. Total cell phosphoamino acids were prepared using a 1-h acid hydrolysis, and equal numbers of counts were analyzed as described previously (Cooper et al., 1983).

[³⁵S]Methionine Labeling of Cells

Swiss 3T3 fibroblasts were labeled with [³⁵S]methionine (50 μ Ci/ml; ICN

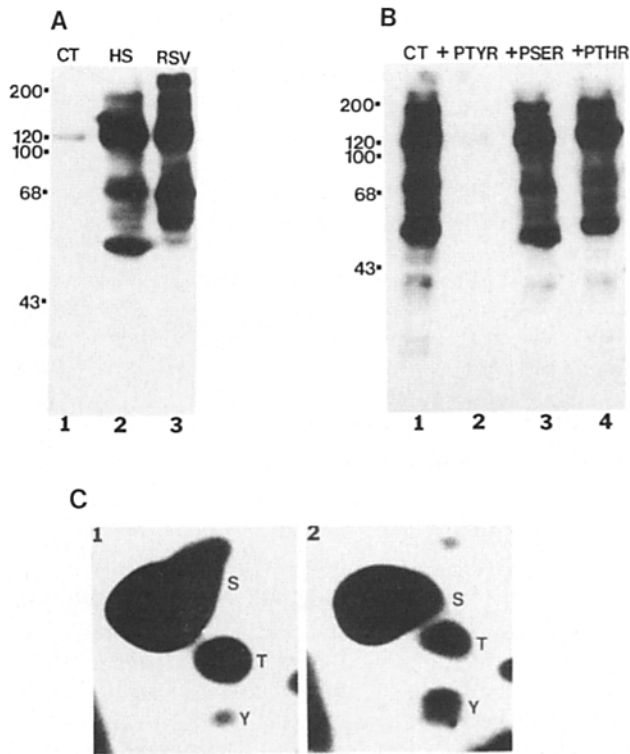


Figure 1. Level of heat-shock-induced protein tyrosine phosphorylation and specificity of antiphosphotyrosine antibodies. (A) Comparison of control Swiss 3T3 fibroblasts (lane 1, no heat shock), heat-shocked Swiss 3T3 fibroblasts (lane 2, 1 h, 45°C), and Rous sarcoma virus-transformed 3T3 fibroblasts (lane 3) by immunoblotting with antibodies specific for phosphotyrosine. (B) Immunoblotting of heat-shocked Swiss 3T3 fibroblasts with antiphosphotyrosine antibodies alone (lane 1) or in the presence of 10 mM phosphotyrosine (lane 2), 10 mM phosphoserine (lane 3), or 10 mM phosphothreonine (lane 4). (C) Total cell protein phosphoamino acid analysis of non-heat-shocked (1) and heat-shocked (2) Swiss 3T3 fibroblasts.

Biomedicals Inc.) for 30 min at 37°C and then incubated at 37 or 45°C for 1.5 h. After electrophoresis, the gels were fixed overnight in 25% isopropanol, 10% acetic acid, treated with EN³HANCE (New England Nuclear, Boston, MA), dried, and autoradiographed.

Immunofluorescence Microscopy

The preparation of the cells and the labeling with the antiphosphotyrosine antibodies and nitrobenzoxadiazole-phalloidin (Molecular Probes Inc., Junction City, OR) was carried out as previously described (Maher and Pasquale, 1988).

Results

The induction of protein tyrosine phosphorylation by heat shock was characterized in both mammalian and chicken cells using high affinity polyclonal antibodies specific for phosphotyrosine residues in immunoblotting experiments. Swiss 3T3 fibroblasts heat shocked at 45°C were probed with antiphosphotyrosine antibodies in the immunoblot of Fig. 1 A, along with non-heat-shocked Swiss 3T3 fibroblasts and Rous sarcoma virus-transformed 3T3 cells. The heat-shocked fibroblasts have high levels of protein tyrosine phosphorylation, similar to those of transformed cells. The specificity of the antibodies for protein phosphotyrosine residues in cells exposed to heat shock is shown in Fig. 1 B. Phosphotyrosine inhibited the reaction of the antibodies with the phosphotyrosine-containing proteins in the immunoblot of heat-shocked cells, whereas the same concentration of phosphothreonine or phosphoserine had no effect. The presence of increased protein tyrosine phosphorylation in cells exposed to heat shock was confirmed by two-dimensional phosphoamino acid analysis of total cellular protein from Swiss 3T3 fibroblasts labeled with [³⁵P]orthophosphate in phosphate-free medium and cultured at 37°C or exposed to 46°C for 1 h as shown in Fig. 1 C. It should be noted that the increase in protein tyrosine phosphorylation induced by heat shock is underestimated when measured by phosphoamino acid analysis. In control immunoblotting experiments (not shown), the

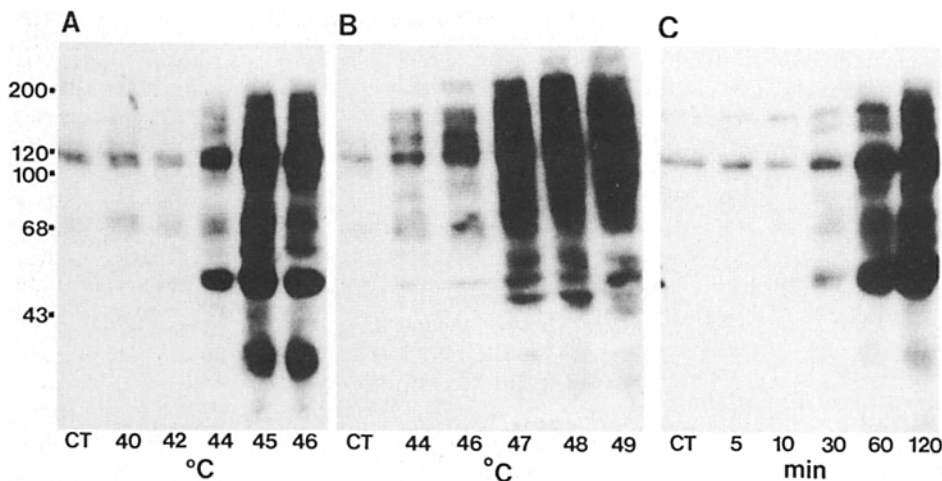


Figure 2. Temperature and time dependence of heat-shock induction of protein tyrosine phosphorylation as studied by immunoblotting with rabbit antiphosphotyrosine antibodies. Cells were incubated in a water bath adjusted to 40–49°C. For the time course studies the culture medium was prewarmed to the desired temperature. (A) Temperature dependence of protein tyrosine phosphorylation in heat-shocked Swiss 3T3 fibroblasts. Cells were heat shocked for 1.5 h at each temperature. (B) Temperature dependence of protein tyrosine phosphorylation in heat-shocked chicken embryo fibroblasts. Cells were treated for 1.5 h at each temperature. (C) Time dependence of protein tyrosine phosphorylation in Swiss 3T3 fibroblasts heat shocked at 45°C.

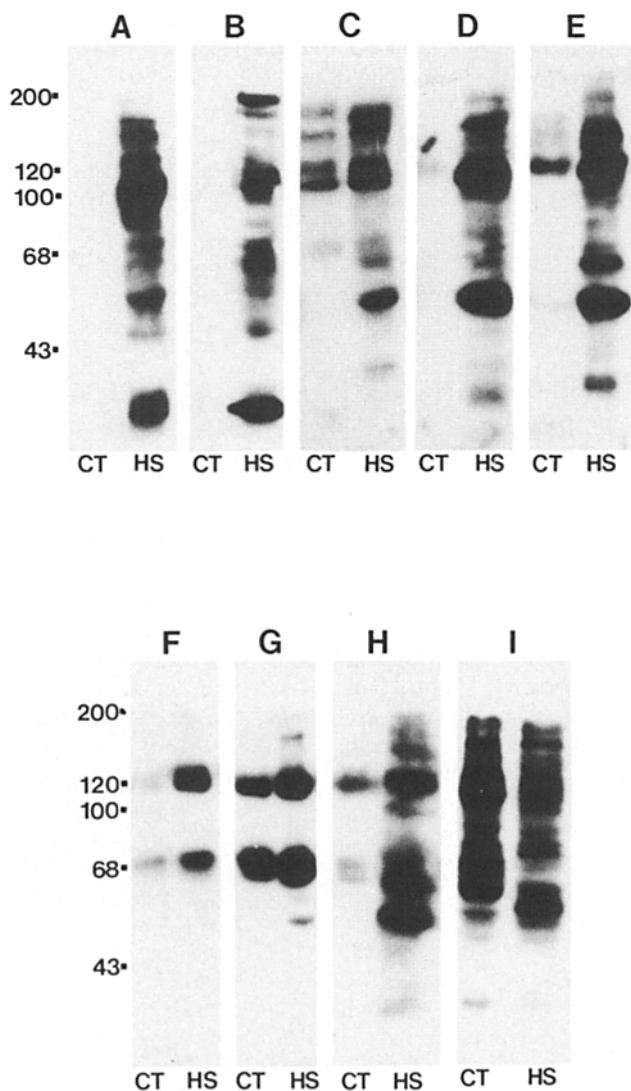


Figure 3. Effect of heat shock on protein tyrosine phosphorylation in different cultured cell lines. All cells were grown at 37°C and were heat shocked for 1.5 h at 45–46°C. Immunoblotting with rabbit antiphosphotyrosine antibodies of extracts from (A) Madin-Darby canine kidney cells; (B) potoroo PTK2 cells; (C) rat L6 cells; (D) rat PC12 cells; (E) mouse N2A cells; (F) rat REF-52 cells; (G) rat NRK cells; (H) quiescent Swiss 3T3 fibroblasts; (I) Rous sarcoma virus-transformed 3T3 fibroblasts.

increase in protein tyrosine phosphorylation after heat shock appeared considerably lower in cells exposed to elevated temperatures in phosphate-free medium (necessary for the efficient ^{32}P -labeling of the phosphoamino acids to be analyzed) than in cells heat shocked in the complete medium used in all other experiments.

The temperature dependence of protein tyrosine phosphorylation in Swiss 3T3 fibroblasts and chicken embryo fibroblasts is illustrated in Fig. 2, A and B, respectively. Only low levels of protein tyrosine phosphorylation were observed in both cell types incubated at temperatures <44°C for 1.5 h. At these temperatures the majority of the phosphotyrosine was associated with a protein band of 120 kD. When the Swiss 3T3 fibroblasts were incubated at $\geq 44^\circ\text{C}$ for 1.5 h, a

large increase in their protein tyrosine phosphorylation was observed (Fig. 2 A). The most prominent phosphotyrosine-containing proteins in the heat-shocked Swiss 3T3 fibroblasts had molecular masses of 120 and 55 kD. The tyrosine phosphorylation of a 30-kD protein band appeared to increase only at the higher temperatures. A number of less prominent phosphotyrosine-containing proteins ranging in molecular masses from 40 to 200 kD were also detectable. In chicken embryo fibroblasts, protein tyrosine phosphorylation increased slightly when the cells were incubated at 44–46°C, but rose to high levels when the cells were incubated at $\geq 47^\circ\text{C}$ (Fig. 2 B). Exposure of the chicken fibroblasts to temperatures from 44 to 46°C produced an increase in the level of phosphotyrosine associated with a 120-kD protein band. Chicken cells incubated at $\geq 47^\circ\text{C}$ also showed phosphotyrosine-containing proteins ranging from 40 to 220 kD. Fig. 2 C shows the time course of induction of tyrosine phosphorylation in Swiss 3T3 fibroblasts at 45°C. The increase in protein tyrosine phosphorylation was not apparent until 30 min after the shift to 45°C and was only fully developed after 1 h at 45°C. The time course of the increase in protein tyrosine phosphorylation in chick fibroblasts at 47°C was similar to that observed with Swiss 3T3 fibroblasts at 45°C (not shown). Thus, the heat-shock-induced increase in protein tyrosine phosphorylation occurs much more slowly (plateau at 60 min) than the increase observed when quiescent cells are stimulated by some growth factors (peak at 2–5 min; Pasquale et al., 1988). For both cell types the temperatures required for induction of extensive protein tyrosine phosphorylation were 2°C higher than those required for induction of heat shock protein synthesis. In addition, sodium arsenite, another inducer of heat-shock proteins, did not increase the levels of protein tyrosine phosphorylation (not shown). The level of protein tyrosine phosphorylation remained elevated for several hours after heat shock (not shown).

To determine whether induction of extensive protein tyrosine phosphorylation by heat shock is a general phenomenon we incubated a number of permanent cell lines at 45–46°C for 1.5 h. The results obtained by immunoblotting both the untreated and heat-treated cells with antibodies specific for phosphotyrosine are shown in Fig. 3. Low levels of protein tyrosine phosphorylation were detected in almost all the cell lines examined at 37°C and highly enhanced levels of phosphorylation were detected after heat shock. The cell lines examined include epithelial-like cells (Madin-Darby canine kidney, PTK2), nerve cells (neuroblastoma N2A, PC12 cells), muscle cells (L6), and rat fibroblasts (REF-52, NRK). Most of these cell lines, as well as the Swiss 3T3 fibroblasts and the chicken embryo fibroblasts, exhibited large increases in tyrosine phosphorylation of proteins with molecular masses of 120, 55, and, frequently, 30 kD. NRK rat fibroblasts were the only exception, since they did not show a large increase in protein tyrosine phosphorylation. The increase in protein tyrosine phosphorylation after heat shock was also observed in Swiss 3T3 fibroblasts made quiescent by serum deprivation before and during the heat shock (Fig. 3 H), indicating that growth factors were not required. The pattern of phosphotyrosine-containing protein bands in the immunoblot of heat-shocked quiescent Swiss 3T3 fibroblasts did not resemble that seen in immunoblots of quiescent Swiss 3T3 fibroblasts stimulated with any of a number of different growth factors (Pasquale et al., 1988).

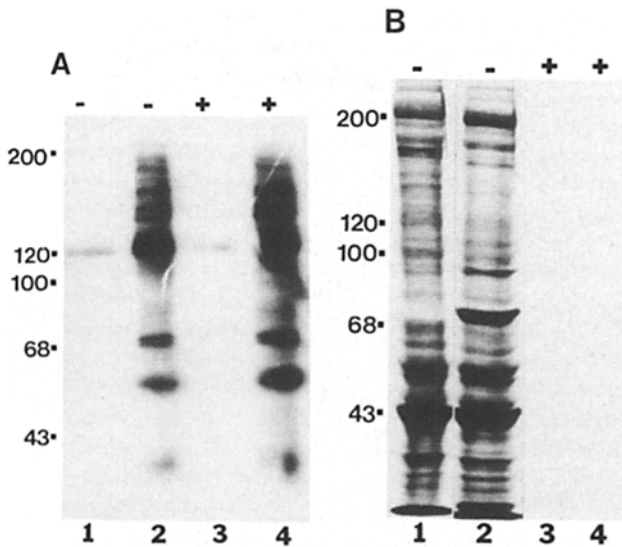


Figure 4. Effect of protein synthesis inhibitors on heat-shock-induced phosphorylation of proteins on tyrosine in Swiss 3T3 fibroblasts. (A) Immunoblot, with antibodies specific for phosphotyrosine, of Swiss 3T3 fibroblasts incubated at 37°C in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of cycloheximide (80 μ g/ml) for 60 min and then incubated at 37°C (lanes 1 and 3) or 45°C (lanes 2 and 4) for 1.5 h. (B) Autoradiograph of Swiss 3T3 fibroblasts incubated at 37°C for 30 min in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of cycloheximide (80 μ g/ml), labeled with [³⁵S]methionine for 30 min at 37°C and then incubated at 37°C (lanes 1 and 3) or 45°C (lanes 2 and 4) for 1.5 h. To visualize the ³⁵S-labeled bands in lane 1, a shorter exposure is shown than that used for lanes 2–4.

Fibroblasts transformed by Rous sarcoma virus had high levels of protein tyrosine phosphorylation at 37°C as compared to their nontransformed counterparts. The elevated protein tyrosine phosphorylation induced by Rous sarcoma virus transformation was not further increased by heat shock (Fig. 3 I).

To determine if protein synthesis is required for heat-shock-stimulated tyrosine phosphorylation, Swiss 3T3 fibroblasts were treated for 30 min with cycloheximide and then heat shocked at 45°C for 1.5 h. As shown in Fig. 4 A, similar increases in protein tyrosine phosphorylation occurred in the absence (–) and in the presence (+) of cycloheximide. Inhibition of protein synthesis alone does not induce protein tyrosine phosphorylation (Fig. 4 A, lanes 2 and 4). Fig. 4 B shows the synthesis of heat-shock proteins in Swiss 3T3 fibroblasts labeled with [³⁵S]methionine in the absence of cycloheximide (lane 2) and the lack of protein synthesis in cells treated with cycloheximide (lane 4).

The heat-shock-induced increase in protein tyrosine phosphorylation could be caused by an inactivation of phosphotyrosine-specific phosphatases rather than by an activation of protein tyrosine kinases. To test this hypothesis chicken embryo fibroblasts were treated for 1 h at 37°C with 50 or 100 μ M sodium orthovanadate, a treatment which was found to effectively inhibit phosphotyrosine phosphatases in cultured cells (Yonemoto et al., 1987). As shown in Fig. 5, the treatment with orthovanadate had little effect on protein tyrosine phosphorylation in these cells (lanes 1–3), confirming the

finding that normal cells have very low levels of phosphotyrosine phosphatase activity (Yonemoto et al., 1987). Treatment of chicken embryo fibroblasts with 50 or 100 μ M orthovanadate during the heat treatment potentiated the increase in protein tyrosine phosphorylation (Fig. 5, lanes 4–6), indicating that low but detectable levels of phosphotyrosine phosphatase activity are still present in chicken embryo fibroblasts after exposure to heat. Thus, an inactivation of phosphotyrosine phosphatases does not appear to be the major factor responsible for the heat-shock-induced increase in protein tyrosine phosphorylation.

We have previously shown that the phosphotyrosine-containing proteins detectable by immunofluorescence microscopy in normal cells are localized to the focal adhesions (Maher et al., 1985). The focal adhesions are discrete, plaque-like regions at the periphery of the ventral cell surface where the cell most closely approaches the substratum and are the sites inside the cell where the F-actin-containing microfilaments terminate. To determine if the increase in protein tyrosine phosphorylation observed in heat-shocked cells occurred at these sites we examined cells immunolabeled with the antiphosphotyrosine antibodies by immunofluorescence microscopy as shown in Fig. 6. Chicken embryo fibroblasts maintained at 37°C were labeled only faintly at the focal adhesions with the antiphosphotyrosine antibodies (Fig. 6 A), whereas cells exposed to 47°C for 30 and 60 min exhibited a large increase in focal adhesion labeling (Fig. 6, C and E). In addition, from these experiments it was determined that all the cells exposed to the heat shock exhibited a similar increase in protein tyrosine phosphorylation (not shown). By double labeling the chicken embryo

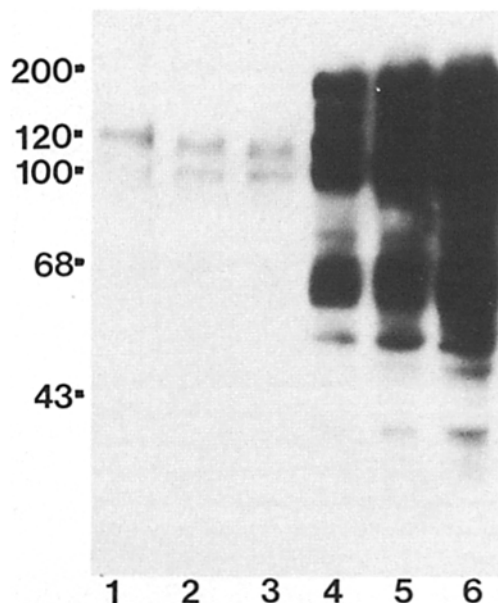


Figure 5. Effect of vanadate on protein tyrosine phosphorylation in chicken embryo fibroblasts. Immunoblot, with antibodies specific for phosphotyrosine, of control cells (lanes 1–3) and cells heat shocked for 1 h at 47°C (lanes 4–6) in the absence (lanes 1 and 4) and presence of 50 μ M (lanes 2 and 5) or 100 μ M (lanes 3 and 6) sodium orthovanadate.

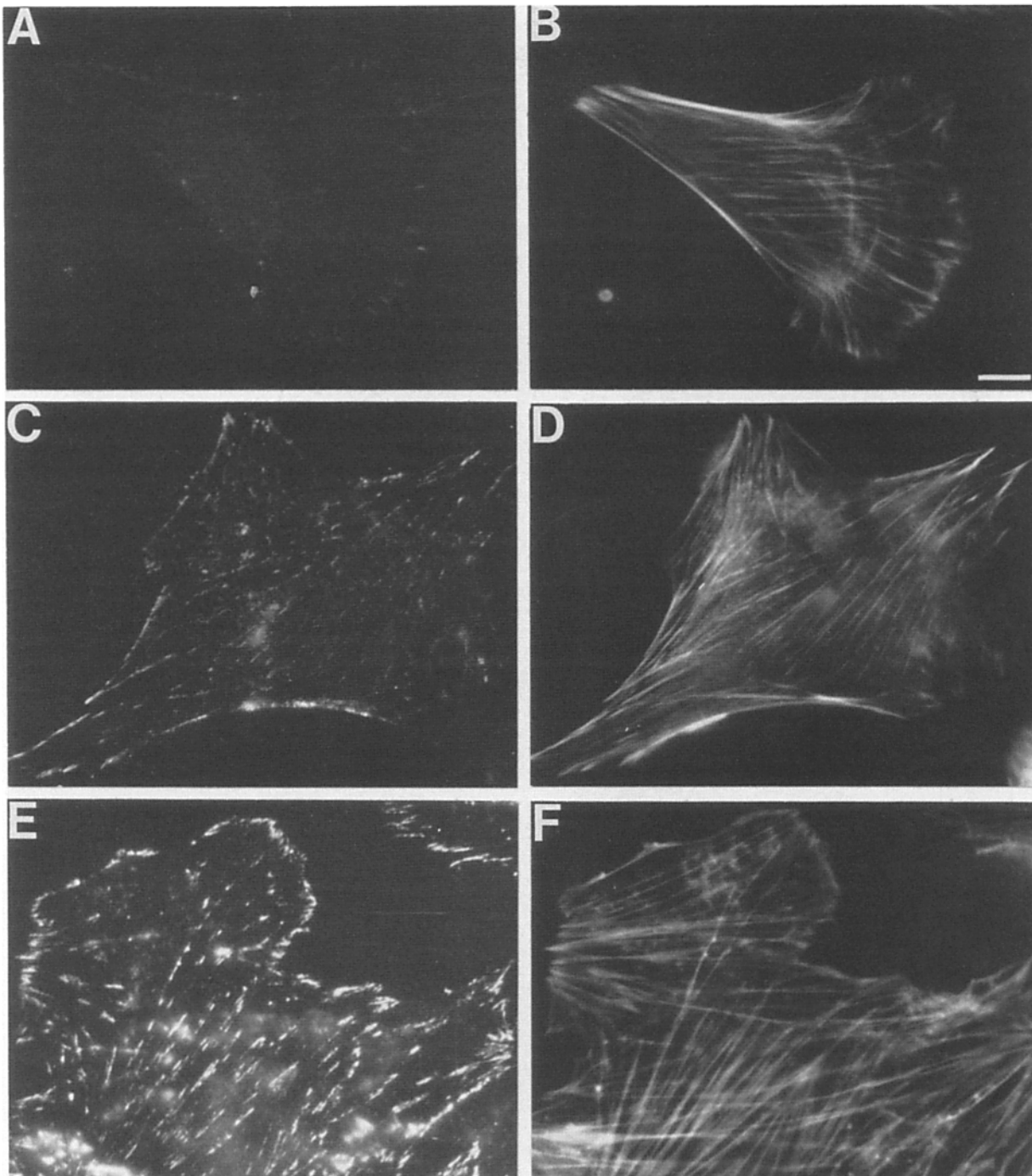


Figure 6. Indirect immunofluorescence labeling of chicken embryo fibroblasts with rabbit antiphosphotyrosine antibodies followed by rhodamine-conjugated goat anti-rabbit IgG (*A*, *C*, and *E*) and nitrobenzoxadiazole-phalloidin to label F-actin (*B*, *D*, and *F*). (*A* and *B*) Control cells; (*C* and *D*) cells heat shocked at 47°C for 30 min; and (*E* and *F*) cells heat shocked at 47°C for 60 min. The exposure times for the film used in this experiment were shorter than those used previously (Maher et al., 1985) so the labeling in the control cells appears less evident. Bar, 1 μ m.

fibroblasts with nitrobenzoxadiazole-phalloidin the organization of the actin-containing microfilaments during the heat treatment could be monitored (Fig. 6, *D* and *F*). The microfilaments (and the overall cellular morphology) appeared to remain intact during the heat shock.

To determine the viability of the cells after the heat shock,

chicken embryo fibroblasts were labeled with trypan blue immediately after a 30- or 60-min heat shock at 47°C, as well as 24 h later. Over 98% of the cells appeared viable immediately after the heat shock. After 24 h, 90% of the cells treated for 30 min and 75% of the cells treated for 60 min were still viable.

Discussion

The results presented here demonstrate that heat shock can induce high levels of protein tyrosine phosphorylation in cultured cells. The proteins previously reported to have altered levels of phosphorylation after heat shock were involved in protein transcription (Glover et al., 1981; Pekkala et al., 1984) or translation (Glover, 1982; Scharf and Nover, 1982; Olsen et al., 1983; Duncan and Hershey, 1984; Pekkala and Silver, 1987), suggesting that phosphorylation may play a role in regulating the changes in protein synthesis that occur after heat shock. However, it is unlikely that these proteins correspond to the major phosphotyrosine-containing proteins we observe, since the molecular masses are not the same.

Some of the heat shock proteins are themselves phosphoproteins (for review see Welch, 1987). However, in our studies, inhibition of heat-shock protein synthesis by cycloheximide did not detectably affect the increase in protein tyrosine phosphorylation after heat shock, suggesting that the heat-shock proteins are not among the proteins which become extensively phosphorylated on tyrosine. The finding that cycloheximide does not block the increase in protein tyrosine phosphorylation also suggests that heat-shock-induced proteins are not responsible for the increase in protein tyrosine phosphorylation.

The mechanism whereby heat shock stimulates protein tyrosine phosphorylation is not known at this time. However, it is clear from the results presented here that the increase is not due to a general inactivation of phosphotyrosine phosphatases. In fact, inhibition of the very low levels of phosphotyrosine phosphatase activity in normal cultured cells does not cause large increases in the extent of protein tyrosine phosphorylation. This indicates that the tyrosine kinase activity in cultured cells must also be low, since protein tyrosine phosphorylation is barely detectable. The stimulation of protein tyrosine phosphorylation is most likely due to an increase in tyrosine kinase activity after heat shock. Protein tyrosine kinases are presumably present and regulated in cultured cells and are activated by exposure to heat. This could be brought about in several possible ways. For instance, in the case of the growth factor receptors, the conformation of the protein appears to regulate tyrosine kinase activity such that the kinase is only active after the growth factor binds. Heat may activate endogenous tyrosine kinases by altering their conformation. In the case of the c-abl protein the amino terminus has been specifically shown to play a role in down-regulating kinase activity (Ben-Neriah and Baltimore, 1986). Similarly, heat could cause a change in the structure of the amino terminus of tyrosine kinases leading to an increase in tyrosine kinase activity. Serine- and threonine-specific protein kinases do not appear to be activated to the same extent as tyrosine kinases by heat, since the two-dimensional phosphoamino acid analysis of total cellular proteins did not show an increase in phosphorylation of serine and threonine relative to control cells. Another possible explanation comes from the finding of proteins which can bind to tyrosine kinases and inhibit their activity. For example, one of the constitutively expressed heat-shock proteins, hsp 90, has been found to bind to the transforming proteins of several oncogenic viruses that encode tyrosine kinases, including Rous sarcoma virus, Fujinami sarcoma virus, and

PRCII avian sarcoma virus (Brugge, 1986; Ziemicki, 1986). The tyrosine kinases that are complexed with hsp 90 appear to lack activity. Although there is no evidence that hsp 90 is involved in regulating the activity of protein tyrosine kinases in normal cells, it is possible that, after heat shock, hsp 90 or other proteins normally involved in a regulatory complex with cellular tyrosine kinases, dissociate from the kinases and thereby lead to their activation.

An alternative explanation, that the increase in protein tyrosine phosphorylation is due to the partial denaturation of a subset of proteins exposing tyrosine residues to kinases, is also possible. However, the finding that not all types of cells show an increase in protein tyrosine phosphorylation after heat shock argues against this explanation. In particular, cells with high basal levels of protein tyrosine phosphorylation, and therefore perhaps fully activated tyrosine kinases, show no phosphotyrosine increase after heat shock, which would not be expected if general protein denaturation was involved.

The precise role increased levels of protein tyrosine phosphorylation may play in the heat-shock response is unclear at this time. However, some hypotheses are ruled out by our data. Phosphorylation on tyrosine is not likely to be involved in regulating the synthesis or function of heat-shock proteins, since the temperatures required to induce tyrosine phosphorylation are 2°C above those required for the induction of the heat-shock proteins. Although relatively high temperatures are required, the increase in protein tyrosine phosphorylation is also unlikely to be involved in cell death, since the majority of the cells can remain viable under conditions which generate substantial increases in protein tyrosine phosphorylation.

The results presented show that the induction of protein tyrosine phosphorylation by heat shock is a general phenomenon, since it was observed in many different cell types. In addition, as shown using immunofluorescence microscopy, all the cells exposed to heat shock, rather than just a small percentage of the population, undergo similar increases in protein tyrosine phosphorylation.

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